

The Fanconi Anemia Proteins FANCD2 and FANCI Interact and Regulate Each Other's Chromatin Localization*

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Background: Fanconi anemia (FA) is a genetic disease of birth defects, bone marrow failure, and cancer.

Results: FANCD2 and FANCI bind and regulate each other's chromatin localization.

Conclusion: Rather than FANCD2 being upstream of FANCI, the two proteins interact in a coordinated set of actions depending on both proteins.

Significance: FA genes are mutated in many cancers, and five FA genes are familial breast cancer genes.

Fanconi anemia is a genetic disease resulting in bone marrow failure, birth defects, and cancer that is thought to encompass a defect in maintenance of genomic stability. Mutations in 16 genes (*FANCA*, *B*, *C*, *D1*, *D2*, *E*, *F*, *G*, *I*, *J*, *L*, *M*, *N*, *O*, *P*, and *Q*) have been identified in patients, with the Fanconi anemia subtype J (FA-J) resulting from homozygous mutations in the *FANCI* gene. Here, we describe the direct interaction of FANCD2 with FANCI. We demonstrate the interaction of FANCD2 and FANCI *in vivo* and *in vitro* by immunoprecipitation in crude cell lysates and from fractions after gel filtration and with baculovirally expressed proteins. Mutation of the monoubiquitination site of FANCD2 (K561R) preserves interaction with FANCI constitutively in a manner that impedes proper chromatin localization of FANCI. FANCI is necessary for FANCD2 chromatin loading and focus formation in response to mitomycin C treatment. Our results suggest not only that FANCD2 regulates FANCI chromatin localization but also that FANCI is necessary for efficient loading of FANCD2 onto chromatin following DNA damage caused by mitomycin C treatment.

Fanconi anemia (FA)³ is a genetic disease, characterized by chromosomal instability, bone marrow failure, congenital defects, and cancer susceptibility (1) (2). The well documented association of FA with DNA damage hypersensitivity has led investigators to classify FA as a DNA repair defect disorder.

Sixteen FA genes have been identified: *FANCA*, *B*, *C*, *D1*, *D2*, *E*, *F*, *G*, *I*, *J*, *L*, *M*, *N*, *O*, *P*, and *Q*, but only a few of their sequences have yielded clues about their functions (3–8). Biochemical studies of these proteins have shown that a “core com-

plex,” which includes *FANCA*, *B*, *C*, *E*, *F*, *G*, *L*, and *M*, is activated upon activation of the DNA damage response, typically during the S phase (9–13). This complex translocates from the cytoplasm to the nucleus, where it participates in the monoubiquitination of FANCD2 (14, 15). Recent evidence suggests that the FANCL subunit is the direct E3 ligase for FANCD2. Shortly thereafter, monoubiquitinated FANCD2 is seen at nuclear repair foci, along with BRCA1, RPA, PCNA, RAD51, and others (16–19).

FA has been linked with the BRCA pathway, because FANCD2 has been shown to interact with BRCA1 and BRCA2 (17, 20), and the familial breast cancer genes *BRCA2* and *PALB2* have been demonstrated to be the FA genes *FANCD1* and *FANCN*, respectively (21–23). The BRCA1-associated helicase, BACH1/BRIP1, was identified as the gene encoding *FANCI* (24–27). A monoallelic missense mutation, R798X, leads to familial breast cancer (28), whereas biallelic R798X leads to FA in 80% of FA patients with the J subtype (FA-J) (26). Studies have shown that cells from patients with FA-J exhibit normal FANCD2 monoubiquitination, and it is hypothesized that the function of FANCI is downstream of this event in the FA pathway (24–26). Although FANCI is thought to be downstream of FANCD2, it is not clear how FANCI is regulated by FANCD2 in the FA pathway. A 2010 paper (29) in part noted co-localization of FANCD2 and FANCI and dependence of FANCD2 foci formation upon FANCI. However, it did not assess interaction of the proteins, did not convincingly show foci formation effects, and did not show how each protein affects chromatin localization.

In this paper, we describe the interaction of nonubiquitinated and ubiquitinated FANCD2 with FANCI. We show by immunoprecipitation, immunofluorescence microscopy and chromatographic analysis of FA protein complexes that FANCI-FANCD2 binding occurs primarily in the absence of induced DNA damage. Mutation analysis shows that the FANCI-FANCD2 interaction is independent of a functional monoubiquitination site, demonstrating that interaction is reduced upon monoubiquitination. Further, FANCI and FANCD2 regulate each other's proper chromatin loading.

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³ The abbreviations used are: FA, Fanconi anemia; MMC, mitomycin C.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells were cultured in DMEM containing 10% fetal bovine serum (Biowest, Miami, FL) and penicillin/streptomycin (Invitrogen). EUFA0030 TERT fibroblasts (kindly provided by H. Joenje) and EUFA0030 TERT FANCI corrected fibroblasts were cultured in RPMI (Invitrogen) with 20% fetal bovine serum. FA-D2 mutant cells PD20, PD20 Flag-FANCD2 and FA-A mutant cells GM6914 and GM6914 Flag-FANCA were cultured in DMEM containing 15% fetal bovine serum and penicillin/streptomycin (30, 31).

Immunoprecipitation—HeLa cells, GM6914 FA-A mutant cells corrected or not with pMMP-Flag-FANCA, and PD20 mutant FA-D2 cells transduced with pMMP-Flag-FANCD2 wild type or FANCD2(K561R) were cultured on 10 cm plates and treated with or without 0.5 μ M mitomycin C (MMC) overnight prior to harvest. Following PBS wash, 1 ml of the radioimmune precipitation assay buffer (150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) was added to cells. Extracts were cleared by centrifugation at 13,000 rpm for 15 min at 4 °C. Supernatants were saved, and equal amounts of extract were used for each immunoprecipitation, with 1 μ g of antibody (FANCD2 H-300, or normal rabbit IgG; Santa Cruz, Santa Cruz, CA) or 2 μ l of Brip1/FANCI rabbit polyclonal antiserum (Novus Biologicals, Littleton, CO). Immunoprecipitations were then performed as previously described (30, 31).

Immunoblotting—Protein samples were suspended in SDS loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 10% β -mercaptoethanol) and boiled for 5 min. Because FANCI and FANCD2 have similar molecular masses (140 and 155 kDa, respectively), we split each sample for immunoblot into two equal aliquots for blotting with either the FANCI or FANCD2 antibodies. Samples were run on 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked in TBS-T (TBS + 0.1% Tween 20) with 10% milk for 1.5 h and incubated in primary antibody (FANCD2 H-300 1:1000 or BRIP1/FANCI rabbit polyclonal antibody 1:2000) for 1 h at room temperature in TBS-T + 1% milk. After washing, membranes were incubated in secondary antibody (ECL anti-rabbit IgG, HRP; Amersham Biosciences) 1:5000 in TBS-T + 1% milk) for 1 h at room temperature. Blots were washed in TBS-T three times for 5 min and developed by chemiluminescence (Supersignal West Pico Kit or Supersignal West Femto kit; Pierce). ImageJ software was used to quantify bands on the blot.

In Vitro Immunoprecipitation—Recombinant FLAG-FANCI baculovirus, kindly provided by S. Cantor (25), was used to infect High Five cells. The protein was isolated using anti-FLAG M2 affinity gel (Sigma) as described (25) and further purified in a Mono S column (Amersham Biosciences). Recombinant Zz-tagged FANCD2 baculovirus, kindly provided by A. D'Andrea (Dana-Farber Cancer Institute) (32), was used to infect High Five cells. The protein was isolated using IgG-Sepharose beads and released from the beads using TEV protease. FANCD2 protein was further fractionated in a Mono Q column (Amersham Biosciences). FANCD2 (2 μ g) was incubated with FANCI (2 μ g) in binding buffer (PBS, 1 mM DTT, and 0.01% Igepal) for 30 min on ice. Then the mixture was incubated with

10 μ l of anti-Flag-agarose beads for 1 h at 4 °C with a rocking block. The beads were pelleted by centrifugation, and the supernatant was saved. After washing three times with 100 μ l of binding buffer, the beads were treated with 20 μ l of SDS loading buffer to elute bound proteins. The supernatant (1 μ l), wash (1 μ l), and SDS eluate (1 μ l) were subjected to SDS-PAGE, and protein contents were identified by immunoblot. Immunoblot analysis (as opposed to gel staining) was used because of the closely similar molecular mass of each protein.

Chromatography—FA-D2 PD20 + pMMP-FlagFANCD2 cells were cultured on 15-cm plates. Following PBS wash, whole cell extract was prepared by lysing cells in nondenaturing lysis buffer (1% Triton X-100, 50 mM Tris-Cl, pH 7.4, 300 mM NaCl, 0.5 mM EDTA, 0.02% Na₃N, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate). Extracts were cleared by centrifugation at 14,000 rpm for 15 min at 4 °C. Supernatants were run through a P11 column, and the flow through was loaded on a Superose 6 gel filtration column (GE Healthcare). Fractions were eluted in nondenaturing lysis buffer, collected, and analyzed by SDS-PAGE. Selected fractions were isolated and analyzed by immunoblot for FANCI and FANCD2 fractionation. The fractions containing FANCD2 and FANCI were combined and subjected to FANCD2 immunoprecipitation for FANCD2-FANCI interaction. Fraction sizes on column were standardized using molecular mass standards (GE Healthcare).

Immunofluorescence Microscopy—Cells were plated in 8-well chamber slides and allowed to adhere 24–48 h and then treated with 0.5 μ M MMC for 24 h. Slides were rinsed with PBS and fixed with 4% paraformaldehyde for 5 min at room temperature. Slides were rinsed with PBS, and cells were permeabilized with PBS containing 0.3% Triton X-100 for 5 min at room temperature. After a final rinse with PBS, slides were blocked in antibody/blocking buffer (PBS with 10% normal goat serum (Invitrogen), 0.1% Nonidet P-40) for 30 min at room temperature. Slides were incubated with primary antibody, FANCD2 F117 mAb (Santa Cruz) or normal mouse IgG (Santa Cruz) diluted 1:1000 in antibody buffer (PBS with 0.5% BSA and 0.1% Nonidet P-40) overnight at 4 °C in a humidified chamber. After washing three times for 5 min in wash buffer (PBS + 0.1% Tween 20), slides were incubated in secondary antibody, Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA), diluted 1:1000 in antibody buffer for 1 h at room temperature or Alexa 488-conjugated goat anti-rabbit (Invitrogen) 1:1000 in antibody/blocking buffer for 45 min at room temperature. Slides were washed three times for 5 min in wash buffer and two times for 5 min in PBS and mounted with VectaShield mounting medium with DAPI Hard Set (Vector Laboratories, Burlingame, CA). Slides were analyzed on a Nikon TE2000-E Eclipse inverted fluorescent microscope or a Zeiss LSM 510-UV confocal microscope. Co-localization analysis was performed using associated Zeiss software package, analyzing 30 cells under each condition.

Cell Fractionation—Procedures for permeabilization and subnuclear extraction were described previously (33). In brief, cells in one 15-cm plate were washed once in PBS, harvested by scraping, and centrifuged for 5 min to pellet. The soluble fraction was extracted by suspending the cells in 1 ml of low salt

buffer (10 mM Hepes, pH 7.4, 10 mM KCl, and 50 μ g/ml digitonin) containing protease and phosphatase inhibitors (2 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4) for 15 min at 4 °C. Permeabilized nuclei were recovered by centrifugation at 2000 rpm for 5 min at 4 °C. The supernatant was termed the soluble fraction. The nuclei were washed two additional times in the same buffer. After wash, the nuclei were resuspended in 200 μ l of low salt buffer containing 30 units of DNase I (RNase free; Roche Diagnostics) for 15 min at room temperature and an additional 15 min at 37 °C and then pelleted again. The supernatant was set aside. The pellet was extracted once again with extraction buffer (1% Triton X-100, 50 mM Hepes, pH 7.4, 150 mM NaCl, and 30 mM $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 10 mM NaF, and 1 mM EDTA) containing protease and phosphatase inhibitors for 15 min at 4 °C. Supernatant was collected at 14,000 rpm in a microcentrifuge for 10 min and pooled with the low salt buffer + DNase I supernatant to form the chromatin fraction. Cellular fractions were analyzed by immunoblot.

FANCD2 siRNA Knockdown—HeLa cells were grown to 60% confluency in DMEM with 10% FBS. 60 nM FANCD2 siRNA pool (Dharmacon, siGENOME SMARTpool) and nontargeting control siRNA (Dharmacon, Lafayette, CO) were transfected in HeLa cells using X-tremeGENE siRNA transfection reagent (Roche Diagnostics) according to the manufacturer's instructions. Soluble and chromatin fractions were made from cells 72 h after transfection and analyzed by Western blot.

RESULTS

FANCI Binds to FANCD2—Previous data have shown that FANCD2 monoubiquitination occurs normally in the absence of FANCI, leading to speculation that FANCI acts downstream of FANCD2 in the FA pathway (7, 9, 34). To examine the relationship of FANCD2 and FANCI in the pathway, we wanted to test for the interaction between FANCI and FANCD2. We first prepared whole cell extracts from FA-D2 mutant PD20 cells containing empty pMMP vector or pMMP vector with cDNA for either wild type FANCD2 or the monoubiquitination mutant FANCD2(K561R), treated or not with 0.5 μ M MMC. We immunoprecipitated the extracts with anti-FANCI antibody. Subsequent immunoblotting against FANCD2 showed that FANCD2 co-precipitated with FANCI in both the wild type and monoubiquitination mutant cell extracts (Fig. 1A). Interestingly, upon MMC treatment, the amount of co-precipitated FANCD2 was reduced in the wild type cells, whereas that in the monoubiquitination mutant was unchanged (Fig. 1A, lanes 1–4). The co-precipitation of FANCI with FANCD2 was confirmed by using Flag antibody in Flag-FANCD2-containing cells (Fig. 1B). To rule out the possibility that DNA may play a role in co-precipitation of FANCI with FANCD2, we also treated cell lysates with DNase I before performing immunoprecipitation. No significant difference was observed between IPs with or without DNase I treatment (Fig. 1B). Reduced interaction between FANCD2 and FANCI upon MMC treatment was also confirmed (Fig. 1B, lane 3 versus lane 4).

The FA core complex contains FANCL, which has an E3 motif and is thought to be the putative ubiquitin ligase for FANCD2. To test the dependence of the interaction upon

the FA core complex, we prepared similar extracts from FA-A mutant (GM6914) and corrected cells. Anti-FANCI (Fig. 1C) immunoprecipitation was performed on extracts from untreated and MMC-treated cells. In both mutant and corrected cells, the FANCI-FANCD2 interaction was preserved, but only in corrected cells was the DNA damage dependent decrease seen (Fig. 1C, lane 5 versus lane 6), consistent with the data in Fig. 1A showing this decrease only in cells with wild type FANCD2. The interaction of FANCD2 and FANCI was also detected in HeLa cells along with the DNA damage-inducible decrease (Fig. 1C, lane 1 versus lane 2), suggesting that the interaction is not an artifact of overexpression. Monoubiquitination does not occur in core complex mutants or the FANCD2 (K561R) mutant, in which FANCD2-FANCI interaction is static, thus indicating that monoubiquitination of FANCD2 is necessary for the induced dissociation of the interaction. However, monoubiquitination of FANCD2 is not sufficient for complete dissociation of the interaction, because both forms of FANCD2 can be co-precipitated by FANCI (Fig. 1, A and C). Confirmation of the interaction between FANCI and FANCD2 was further provided by size exclusion chromatography of cell protein extracts and by confocal immunofluorescence microscopy. Both FANCI and FANCD2 co-fractionated in a protein complex of ~669 Da in extracts from PD20 + Flag-FANCD2 cells (Fig. 1D, fractions 21–27), and immunoprecipitation with these fractions showed that FANCI can be pulled down by FANCD2 (Fig. 1D). The confocal immunofluorescence microscopy showed that untreated cells had significantly higher mean co-localization (40%) than the MMC-treated cells (25%) (Fig. 1E), in agreement with immunoprecipitation data (Fig. 1, A–C).

To ascertain whether FANCD2 and FANCI interact directly, we purified baculovirally generated FANCD2 and Flag-FANCI proteins, which were then incubated together *in vitro*. Immunoprecipitation was then performed using anti-Flag agarose beads. The beads were then washed and treated with SDS to elute proteins, and samples of the supernatant, wash, and eluate were loaded on a gel. Immunoblotting revealed that Flag-FANCI co-precipitated FANCD2 (Fig. 1F, lane 8), suggesting that these proteins interact directly and do so in the absence of DNA.

Because FANCI interacts with FANCD2, we tested whether FANCI interacts with the FA core complex. To that end, we examined the interaction of FANCI with the FA-core complex protein, FANCA. Immunoprecipitation of whole cell extract made from GM6914 FA-A cells corrected with FLAG-FANCA did not show that FANCI and FANCA co-precipitated under our experimental condition, even though the interactions of FANCI and MLH1 or FANCA and FANCG were observed (Fig. 1G) as shown in previous publications (35, 36).

FANCD2 Is Required for Proper Chromatin Localization of FANCI and BRCA1—Because FANCI binds to both monoubiquitinated and nonubiquitinated FANCD2 and FANCI was thought downstream of FANCD2, we explored the functional significance of this interaction by studying its impact on FANCI chromatin loading. Extracts from FANCD2 mutant and corrected cells, treated or not with MMC, were assayed by immunoblotting to examine chromatin loading of FANCI. As expected, FANCD2 localized to chromatin in a DNA damage dependent manner (Fig. 2A, lane 2 versus lanes 5 and 8). FANCI



AND

its diminished interaction with FANCI and because nonmonoubiquitinated FANCD2 acts as a negative regulatory factor in sequestering FANCI from chromatin under non-DNA damage conditions. Mutant, nonmonoubiquitinatable FANCD2 thus constitutively sequesters FANCI, preventing FANCI chromatin localization.

Knockdown of FANCD2 Results in Aberrant FANCD1 and BRCA1 Localization—To confirm the behavior of FANCD1 in a FANCD2-negative background, we transfected pooled siRNA oligonucleotides directed against FANCD2 in HeLa cells, which resulted in diminished FANCD2 expression (Fig. 3A, lanes 3, 4, 7, and 8). Immunoblotting for FANCD1 and BRCA1 demonstrated an increase in the chromatin localization of both proteins in the FANCD2-depleted cells under non-DNA damage conditions, further suggesting that FANCD2 acts as a negative regulator for chromatin localization for both proteins (Fig. 3B,

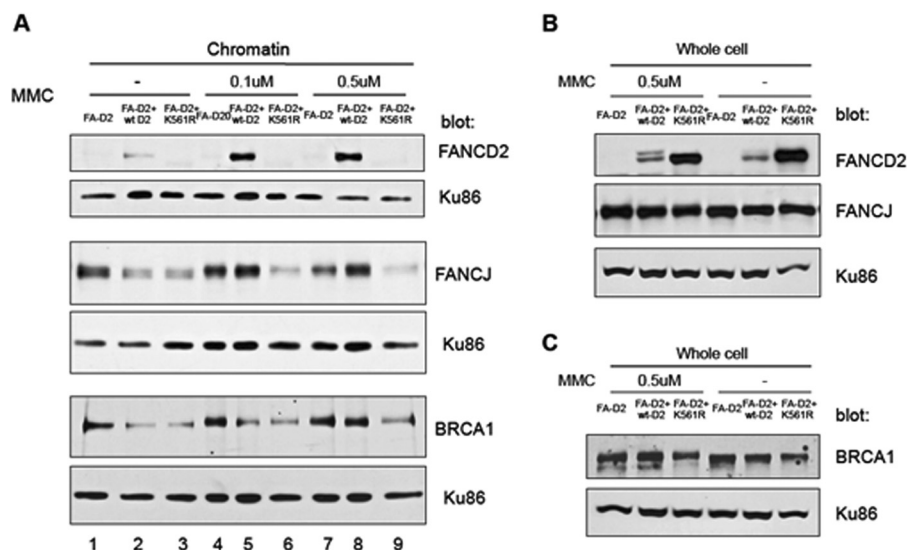


FIGURE 2. **FANCD2 is required for proper chromatin localization of FANCD2 and BRCA1.** A, FA-D2 mutant cells and those transduced with wild type FANCD2 or FANCD2 (K561R) mutant were treated with the indicated dose of MMC, and chromatin extracts were prepared. Immunoblotting revealed that FANCD2 was constitutively present on chromatin in FA-D2 + vector mutant expressing no FANCD2 but not present in FA-D2 + FANCD2 (K561R). FANCD2 chromatin localization was inducible in wild type FANCD2-containing cells. B, whole cell extracts were prepared and analyzed by immunoblotting to demonstrate that similar amounts of FANCD2 were present in each and that FANCD2(K561R) was expressed, even though not present in chromatin in A. C, whole cell extracts were similarly prepared and analyzed by immunoblot to demonstrate similar amounts of BRCA1 present in each.

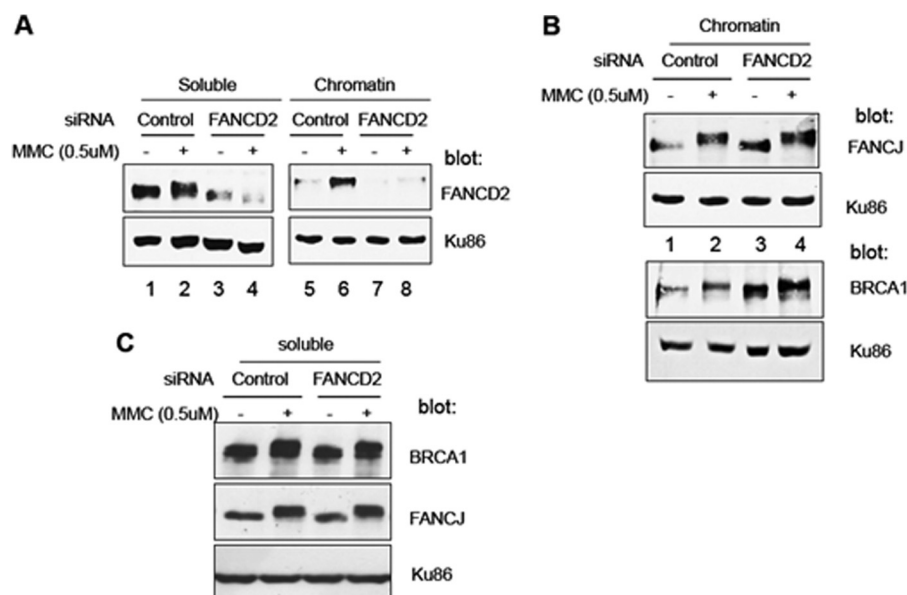


FIGURE 3. **Knockdown of FANCD2 results in aberrant FANCD2 and BRCA1 localization.** A, HeLa cells were transfected with pooled siRNA directed against FANCD2 or luciferase (control). Cells were treated or not with MMC, and extracts were prepared. Immunoblot revealed knockdown of FANCD2 expression (lanes 3 and 4) accompanied diminished chromatin localization (lanes 7 and 8). B, FANCD2 and BRCA1 immunoblotting of extracts from A showed increased localization of both FANCD2 and BRCA1 upon FANCD2 knockdown. C, FANCD2 and BRCA1 immunoblotting of soluble fraction showed similar levels of both proteins in control or FANCD2 knockdown cells.

lane 1 versus lane 3). The levels of FANCD2 and BRCA1 in soluble fraction were not significantly changed (Fig. 3C). Decreased mobility of FANCD2 was evident and consistent with FANCD2 becoming phosphorylated upon DNA damage.

FANCD2 and FANCD2 Chromatin Localization Is Impaired in FA-A and FA-C Core Complex Mutants—Because nonmonoubiquitinated FANCD2 sequesters FANCD2 away from chromatin, we tested FANCD2 localization in FA core complex mutants. In both FA-A and FA-C mutant cells that express nonmonoubiquitinated FANCD2, which fails to localize to chromatin, decreased FANCD2 chromatin localization was observed (Fig. 4,

A and B). This is in contrast to the FANCD2 deletion mutant FA-D2 cell line (Fig. 2A), in which there was no FANCD2 expression (Fig. 4, lanes 1 and 2 versus lanes 3 and 4 and lanes 5 and 6 versus lanes 7 and 8). These data are consistent with those displayed in Fig. 2A in which the FANCD2 (K561R) mutant that fails to become monoubiquitinated sequestered FANCD2 away from chromatin, even under DNA damage conditions.

FANCD2 Regulates FANCD2 Focus Formation—Conversely, we wanted to understand whether FANCD2 was required for FANCD2 localization to chromatin. We first examined the role of FANCD2 in FANCD2 focus formation by immunofluorescence

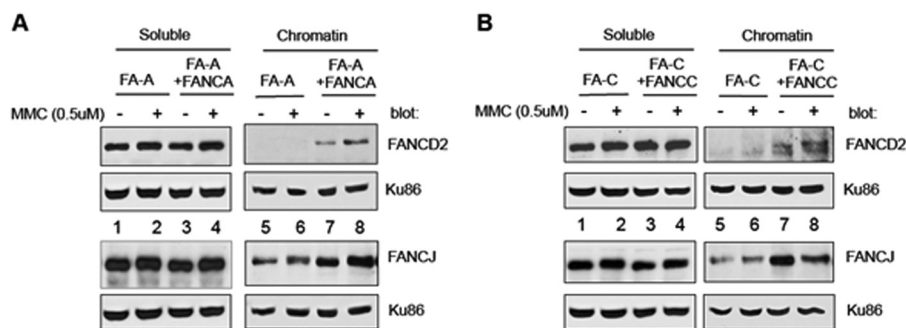


FIGURE 4. **FANCD2 and FANCI chromatin localization is impaired in FA-A and FA-C core complex mutants.** A and B, FA-A mutant (GM6914) and corrected cells (+FANCA) (A) and FA-C mutant (HSC536) and corrected (+FANCC) (B) were treated or not with MMC and subjected to obtain chromatin extraction. Immunoblotting revealed that only corrected cells showed the full amounts of FANCD2 and FANCI chromatin localization after DNA damage.

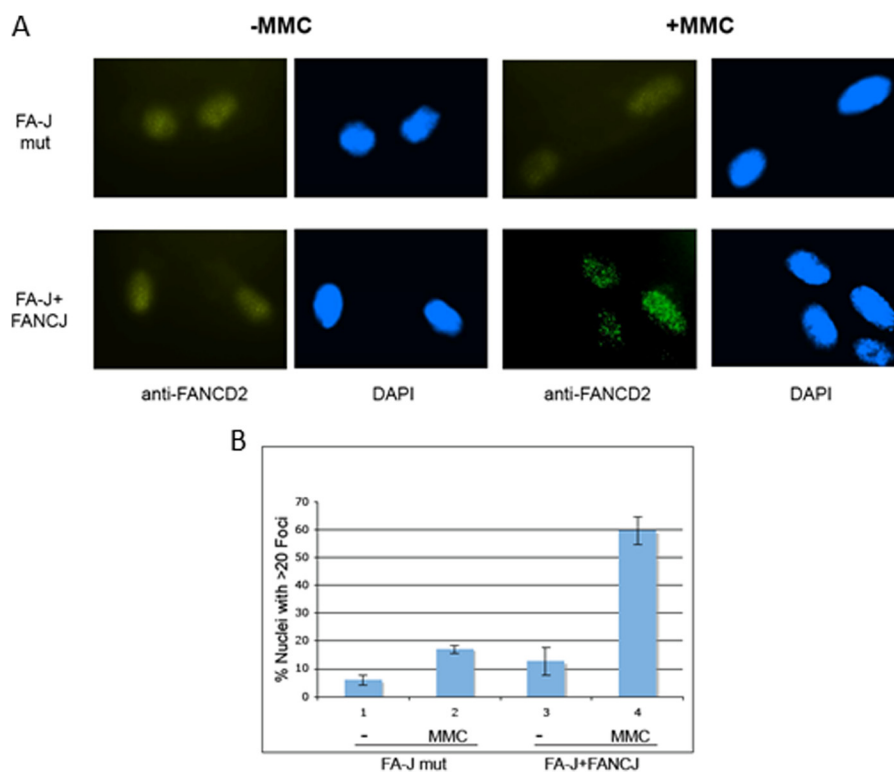


FIGURE 5. **FANCI regulates FANCD2 focus formation.** A, FA-J mutant and corrected (+FANCI) cells were plated and subjected to MMC treatment and then analyzed by immunofluorescence microscopy. B, cells from A were counted, and those with greater than 20 foci per nucleus were scored as positive. The resulting data were represented on a graph, demonstrating that induction of foci was present only in corrected FA-J cells.

microscopy studies of FANCD2 upon DNA damage, using EUFA30 FA-J mutant and corrected. Cells were seeded onto chamber slides and allowed to recover for 24 h and then treated with 0.5 μ M MMC overnight. Following staining for FANCD2, the cells were examined by immunofluorescence microscopy (Fig. 5A). Corrected FA-J cells displayed an increase in FANCD2 focus formation after MMC treatment. Although some foci were still detectable in mutant cells, counts of 100 cells among FA-J mutant and corrected cells demonstrated significantly more corrected cells with FANCD2 foci (Fig. 5B).

FANCI Regulates FANCD2 and BRCA1 Chromatin Loading—Because FANCD2 foci formation is associated with inducible localization to chromatin, we next prepared chromatin or whole cell extracts from FA-J mutant and corrected cells treated or not with MMC to determine the effect of FANCI on FANCD2 chromatin localization. Immunoblotting against

FANCD2 revealed increased MMC-induced FANCD2 localization to chromatin only in the corrected cells (Fig. 6A, lanes 5 and 6). A similar pattern was seen with FANCI, the binding partner of FANCD2, which is also monoubiquitinated. A blot of whole cell extracts showed that FANCD2 was normally monoubiquitinated, as expected, in the FA-J mutant and corrected cells (Fig. 6B). Also as expected, MMC-induced chromatin localization of BRCA1 was reduced in the FA-J mutant cells (Fig. 6C). Again no significant difference in BRCA1 levels was observed in whole cell extracts between FA-J mutant and corrected cells (Fig. 6D).

DISCUSSION

We describe the interaction of FANCI with FANCD2 and characterize the dependence of each on the eventual chromatin localization of the respective protein. We observe that

FANCI and FANCD2 Regulate Each Other

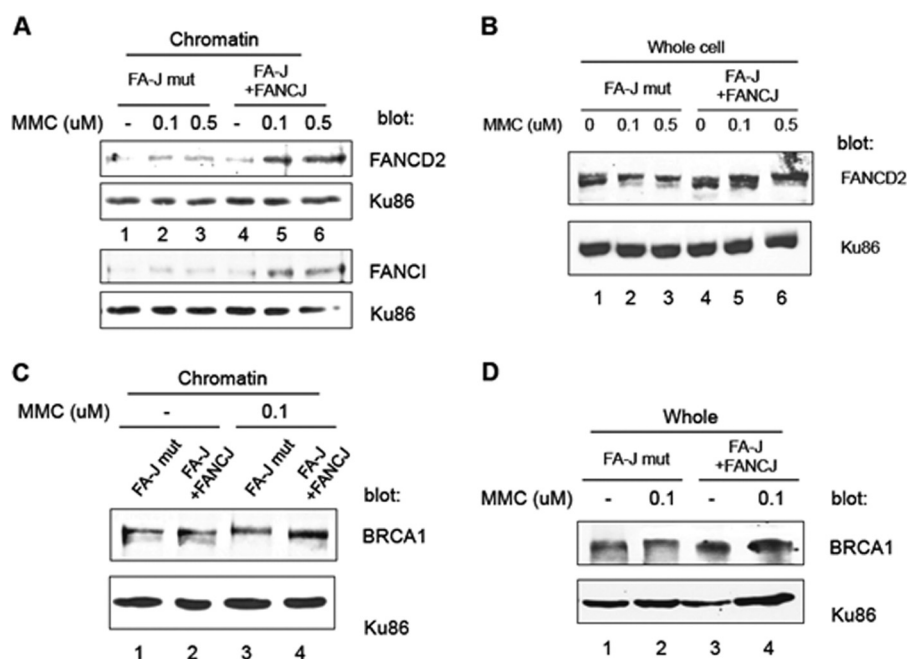


FIGURE 6. FANCI regulates FANCD2 and BRCA1 chromatin loading. A, FA-J mutant and corrected cells were treated with the indicated dose of MMC, and chromatin extracts were prepared. Immunoblotting demonstrated that FANCD2 and FANCI loading onto chromatin was DNA damage-inducible. B, immunoblotting of whole extracts from A demonstrated that FANCD2 monoubiquitination was induced in both mutant and corrected FA-J cells. C, immunoblotting of chromatin extracts from mutant and corrected cells demonstrated inducible localization of BRCA1 in corrected cells. D, immunoblotting of whole extracts demonstrated that BRCA1 protein levels were similar in both mutant and corrected FA-J cells.

FANCD2 directly interacts with FANCI and is a negative regulator of FANCI by virtue of the ability of nonmonoubiquitinated FANCD2 to bind to FANCI, preventing FANCI from localizing to chromatin. The binding of nonmonoubiquitinated FANCD2 to FANCI may also play a role in dissociating FANCI from chromatin after DNA damage is repaired. Monoubiquitination of FANCD2 is tied to eventual FANCI chromatin localization, because monoubiquitinated FANCD2 has lower affinity to FANCI in immunoprecipitation-immunoblotting experiments. Conversely, FANCI is necessary for efficient loading of FANCD2 onto chromatin and for FANCD2 focus formation following MMC-induced DNA damage. Our model in Fig. 7 suggests that activation of FANCD2 via monoubiquitination, and thus eventual localization to chromatin, is a multistep process. In this model, FANCI is necessary for FANCD2 to localize to chromatin after FANCD2 monoubiquitination, thus explaining the co-precipitation of both forms of FANCD2 by FANCI in the pre-DNA damage state.

FANCI was originally postulated to act downstream of FANCD2 in the FA/BRCA DNA repair pathway, because monoubiquitination of FANCD2 occurs in the absence of FANCI (38). We propose that FANCI function is not distinctly downstream of FANCD2 but is cooperative with FANCD2. Monoubiquitination of FANCD2 can be uncoupled from its loading onto chromatin and from repair focus formation, supporting previous findings that monoubiquitination of FANCD2 is necessary but not sufficient for chromatin loading (39). Some detectable FANCD2 foci and chromatin localization was seen even in FA-J mutant cells, but it is markedly diminished in comparison with wild type or corrected cells. Residual FANCI protein or mutant protein with partial function in the mutant cells may account for this.

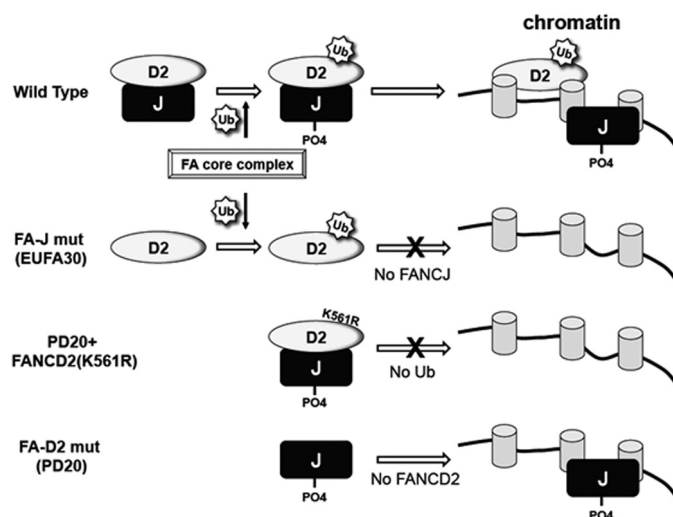


FIGURE 7. Model of FANCD2 and FANCI. FANCD2 binds FANCI. After monoubiquitination and phosphorylation of FANCD2 and FANCI both are loaded onto chromatin. Without ubiquitination, FANCD2 and FANCI remain unbound to chromatin (shown by expression of the FANCD2(K561) mutant but also seen in FA core complex mutants). FANCD2 can be monoubiquitinated by core complex in the absence of FANCI. However, it does not load onto chromatin. In the absence of FANCD2, FANCI can load onto chromatin.

Nonmonoubiquitinated FANCD2 binds to FANCI without DNA damage, suggesting that FANCI may sequester nonmonoubiquitinated FANCD2 in the predamage state, perhaps in a preparatory or staging complex that facilitates rapid activation. This idea is supported by chromatography showing that FANCI and FANCD2 are both found in a 669-kDa complex. Others have not previously shown this interaction, a fact that may be accounted for by formation of different complexes containing additional proteins that block epitopes specific to

immunoprecipitating antibodies, analysis of conditions under which FANCI-FANCD2 interaction is diminished, or different types of lysate preparation with respect to detergent (27).

The findings reported here contrast with those reported by the Andreassen group (29). Close examination of the data presented in that paper in fact demonstrate some foci induction and chromatin localization of FANCD2 in FA-J mutant cells, contrary to the paper's overall conclusion. Our work, on the other hand, demonstrates an almost a complete lack of FANCD2 foci formation and chromatin localization or induction. Technical differences may account for this because our chromatin fractionation technique yields protein fractions containing only monoubiquitinated FANCD2 as opposed to both isoforms seen in the aforementioned paper (25, 29). In addition, our co-immunoprecipitation data are consistent with co-localization predamage and lack of co-localization by confocal microscopy (Fig. 1E). It has long been hypothesized that FANCI functions downstream of FANCD2 monoubiquitination (38), but the demonstration that FANCD2 regulates FANCI chromatin localization provides a mechanism how FANCD2 controls "downstream" FANCI in the FA pathway. The model also predicts that patients with mutations in core complex proteins, representing the bulk of FA individuals, exhibit suboptimal chromatin localization of FANCI because of the association of nonmonoubiquitinated FANCD2 with FANCI.

FANCI and FANCD2 are members of DNA repair protein complexes that may include members such as BRCA1/2, H2AX, and others. In particular, BRCA1 has been identified as binding to and regulating monoubiquitinated FANCD2 (20, 40) and histone H2AX (41). H2AX plays an important role in retaining DNA damage proteins, such as BRCA1, on chromatin. We observe that H2AX signaling is not impaired in FA-D2 mutant cells, suggesting that upstream activation events in FA mutant cells appear to be intact (data not shown). FANCD2 focus formation during DNA damage is dependent upon FANCI (Fig. 5A), similar to how BRCA1 also binds to FANCI (42) to facilitate BRCA1 participation in repair focus formation (37) and DNA repair (42). However, the interaction between BRCA1 and FANCI appears to be ubiquitous and independent of DNA damage (37). FANCI is also known to function independently of BRCA1 in the chicken DT-40 cell system in a manner that implicates it in the FA pathway (24). We show that the association of BRCA1 and FANCI is lower in the complemented FA-D2 cells compared with mutant cells (Fig. 2A). Lower MMC concentrations may result in differential stimulation of such association or localization. These combined data suggest a possible three-way interaction between FANCI, BRCA1, and FANCD2. The regulation of BRCA1 chromatin localization may be multifactorial, because RAP80 forms a complex with BRCA1 but not FANCI and directs BRCA1 chromatin localization (43, 44).

Whereas the DNA helicase activity of FANCI has been demonstrated *in vitro* (45), this activity has yet to be shown in cells, and the possibility remains that the helicase activity of FANCI serves to dissociate protein from DNA, similar to the yeast helicase Srs2 (46, 47). FANCI has also been demonstrated to unwind guanine quadruplex structures (G4-DNA) that can form in tracts of guanines, and a FA-J patient-derived mutant

FANCI protein is unable to unwind these structures *in vitro* (48, 49). It has been speculated that certain interstrand cross-links may preferentially form in these quadruplex structures and that FANCI is required for their resolution. Thus, regardless of the precise mechanism, FANCI may prepare DNA damage sites for the chromatin recruitment of DNA repair factors, such as BRCA1 and monoubiquitinated FANCD2. Consistent with this premise was the demonstration that FANCI functionally interacts with TopBP1, a protein critical for the control of the DNA replication checkpoint (50). The FANCI-TopBP1 interaction was shown to promote chromatin loading of RPA, a requirement for the activation of the apical checkpoint kinase ATR. Therefore, FANCI is important for responding to various forms of replication stress and has a critical role early in DNA replication checkpoint control (51). Given these data, we postulate that FANCI plays a pivotal role in a regulatory pathway that leads to FANCD2 and BRCA1 participation in replication-associated DNA damage repair.

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The Fanconi Anemia Proteins FANCD2 and FANCI Interact and Regulate Each Other's Chromatin Localization

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